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Evaluation of Flowering Cherry Species, Hybrids, and Cultivars Using Simple Sequence Repeat Markers

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Abstract. **Flowering cherries belong to the genus *Prunus*, consisting primarily of species native to Asia. Despite the popularity of ornamental cherry trees in the landscape, most ornamental *Prunus* planted in the United States are derived from a limited genetic base of Japanese flowering cherry taxa. A diverse collection of ornamental *Prunus* germplasm is maintained at the U.S. National Arboretum as part of an ongoing flowering cherry improvement program, but the genetic backgrounds of many trees are unclear. We characterized this germplasm using five simple sequence repeat (SSR) primer pairs, including one chloroplast primer pair. These primers generated 140 unique alleles that were used to assess genetic relationships among species, hybrids, and cultivars in this collection. We found that these markers followed expected Mendelian inheritance from parents to progeny in controlled hybridizations. In general, species clustered according to published taxonomic groupings, including a distinct separation of the ornamental cherries (*Prunus* subgenus *Cerasus* section *Pseudocerasus*) from other subgenera. Individual accessions of several taxa did not cluster with other samples of the species, indicating possible misidentification or interspecific combinations. The resulting information will be useful in guiding decisions on breeding methodology and germplasm preservation.**

Ornamental flowering cherry trees (*Prunus* species) are popular plants for street, commercial, and residential landscapes. Grown primarily for their spring bloom, flowering cherries have been in the United States since the mid-1850s (Faust and Suranyi, 1997), and gained in popularity after the historic Tidal Basin cherries were planted in Washington, DC, in 1912. Over 1 million plants are sold wholesale each year at a value of more than $22 million [U.S. Department of Agriculture (USDA), 2001]. Despite the large number of *Prunus* species with diverse origins and ornamental traits, the most widely cultivated flowering cherry trees planted in the United States represent only a few species, primarily *P. serrulata*, *P. subhirtella*, and *P. ×yedoensis*. The U.S. National Arboretum (USNA) has an ongoing breeding program aimed at broadening this base by developing new cultivars of ornamental cherry with disease and pest resistance, tolerance to environmental stresses, and superior ornamental characteristics. Integral to this breeding program is our flowering cherry germplasm collection, consisting of over 1500 trees representing at least 30 diverse taxa.

The taxonomy and nomenclature of the ornamental flowering cherries is complicated and confusing. Within the genus *Prunus*, the ornamental flowering cherries are in the subgenus *Cerasus* section *Pseudocerasus* (Rehder, 1940; USDA, 2009). The majority of the ornamental species are native to Japan and Korea, where centuries of propagation and cultivation have led to the selection of hundreds of genotypes (Flower Association of Japan, 1982). Confusion over names and origins has arisen due to their long history of cultivation in Japan, including extensive plantings for "cherry viewing," that has resulted in the naturalization of species and selections beyond their native ranges and spontaneous hybridization between native and cultivated species or cultivars (Jefferson and Wain, 1984; Notcutt and Notcutt, 1935). In fact, it has been proposed that species designations for some of the cultivated Japanese flowering cherries be abolished in favor of the designation "Sato-zakura group" (Jefferson and Wain, 1984), which would refer to all cultivated selections of Japanese flowering cherries with uncertain species origins. In this report, we followed the classification system of Rehder (1940) and taxonomic authorities therein or designated by the USDA's Germplasm Resources Information Network (USDA, 2009). Species designations for cultivars are from our accession data or published references with notation indicating which are of unknown specific origin and could be included in the "Sato-zakura group" (Table 1).

The purpose of this study was to use SSR markers to assess genetic relationships in a diverse collection of ornamental *Prunus* germplasm at the USNA. This genetic information has multiple applications: 1) to gain insight into taxonomic relationships in this group, 2) to determine relatedness among parents in our breeding program, 3) to minimize duplicate samples in the germplasm collection, and ultimately 4) to guide breeding decisions to increase the diversity of ornamental cherry cultivars in the landscape.

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Table 1. *Prunus* taxa at the U.S. National Arboretum (USNA) evaluated using five SSR primer pairs. Classification follows Rehder (1940). Symbols beside subgenera are used to provide clarity in Fig. 1. Unless noted in the last column, all accessions except cultivars are derived from seed.

Name (with accession code, if applicable)	Samples (no.)	Collection information and USNA accession no.[z]
Species and cultivars		
Subgenus *Amygdalus* ○		
P. persica 'Harson'	1	DNA from A. Callahan, USDA
Subgenus *Cerasus*		
Section *Phyllomahaleb*		
Prunus maximowiczii (62)	3	1980 Hokkaido, Japan (70773)
Prunus maximowiczii (391)	3	Cheju Island, Korea (59191)
Section *Pseudocerasus*		
P. cerasoides (365)	2	1982 Shizuoka Pref. Japan (50682)
P. campanulata (321)	2	1986 Kukuan Taiwan (58778)
P. campanulata (331)	1	1986 Taiwan (58774)
P. campanulata (418)	1	Scions from Taiwan (69013)
P. cyclamina (152)	1	USNA (34275)
P. incisa (51)	1	1980 Japan (45937)
P. incisa (233)	3	1986 Honshu, Japan (58817)
P. incisa (368)	2	1982 Hokkaido, Japan (50698)
P. incisa f. *yamadei* (183)	3	1982 Japan (50367)
P. incisa 'February Pink'	1	Glenn Dale, MD PI station (47106)
P. ×*kanzakura* Makino (369)	3	1982 Shizuoka Pref., Japan (50703)
P. leveilleana 'Fudan Zakura'[y]	1	Glenn Dale, MD PI station (56415)
P. nipponica (29)	2	1979 Japan (45734)
P. nipponica var. *kurilensis* (211)	3	Hokkaido, Japan (50727)
P. nipponica var. *kurilensis* (371)	2	1982 Hokkaido, Japan (50719)
P. sargentii (63)	2	1980 Hokkaido, Japan (70774)
P. sargentii (79)	2	Hokkaido, Japan (44109)
P. sargentii (299)	2	1986 Fukushima Pref., Japan (58819)
P. sargentii (300)	2	1986 Fukushima Pref., Japan (58820)
P. serrulata 'Amanogawa'[y]	1	USNA (52532)
P. serrulata 'Kwanzan'[y]	1	USNA (70285)
P. serrulata 'Royal Burgundy'[y]	1	USNA (74570)
P. serrulata 'Shirotae'[y]	1	USNA (23899)
P. serrulata var. *lannesiana* 'Superba'[y]	1	USNA (64968)
P. 'Snofozam' (Snow Fountains®)	1	USNA (70284)
P. speciosa (177)	1	1982 Japan (50340)
P. subhirtella 'Autumnalis'	1	Glenn Dale, MD PI station (60609)
P. subhirtella 'Pendula Plena Rosea'	1	USNA (74568)
P. subhirtella (393)	2	1989 Flower Assn., Japan (61383)
P. subhirtella var. *ascendens* (374)	1	1982 Japan (50739)
P. takasagomontana (326)	1	1986 Taiwan (58781)
P. takasagomontana (327)	1	1986 Taiwan (58782)
P. takesimensis (335)	2	1986 Ullung Island, Korea (58789)
P. takesimensis (338)	2	1986 Ullung Island, Korea (58792)
P. takesimensis (339)	2	1986 Ullung Island, Korea (58793)
P. takesimensis (340)	2	1986 Ullung Island, Korea (58794)
P. takesimensis (347)	3	1986 Ullung Island, Korea (58801)
P. verecunda (32)	1	1979 Japan (45737)
P. verecunda (136)	1	USNA (16374)
P. verecunda (394)	2	1989 Akita-ken, Japan (61384)
P. ×*yedoensis* (47)	1	Cutting – Longwood Gardens, PA (70776)
P. ×*yedoensis* (122)	1	Cutting – Tidal Basin, Washington, DC (42006)
P. ×*yedoensis* (123)	1	Cutting – Tidal Basin, Washington, DC (42007)
P. ×*yedoensis* 'Afterglow'	1	USNA (70283)
P. ×*yedoensis* 'Akebono'	1	1985 Cutting – Washington, DC (55534)
Subgenus *Laurocerasus* ■		
P. lusitanica (33)	1	USNA

continued next page

Table 1. Continued.

Name (with accession code, if applicable)	Samples (no.)	Collection information and USNA accession no.[z]
Subgenus *Padus* ◁		
P. buergeriana (329)	1	1986 Cheju Island, Korea (58784)
P. buergeriana (390)	3	1987 Cheju Island, Korea (59190)
P. grayana (109)	1	Glenn Dale, MD PI station (43056)
P. maackii (19)	1	Glenn Dale, MD PI station (70769)
P. maackii (416)	2	Glenn Dale, MD WLPGR (68773)
P. maackii (417)	3	Glenn Dale, MD WLPGR (68811)
P. maackii (470)	1	USNA
P. padus (401)	3	1992 Mt. Odae, Korea (62808)
P. padus (429)	1	Glenn Dale, MD WLPGR (67792)
P. padus 'Wateri' (22)	1	USNA (37041)
P. virginiana 'Schubert'	1	USNA (35892)
Subgenus *Prunus* ●		
Section *Armeniaca*		
P. mume 'Kobai'	1	USNA (59234)
P. mume 'Peggy Clarke'	1	USNA (37157)
Section *Prunocerasus*		
P. maritime	1	USNA (71561)
Section *Prunus*		
P. salicina (430)	3	Glenn Dale, MD WLPGR (67714)
P. salicina var. *mandshurica* (200)	2	USNA (52767)
Hybrids of known origin		
Open pollinated seedling of *P.* 'Kursar' (5006)	1	USNA unnamed hybrid
P. subhirtella 'Autumnalis' × *P. campanulata* (5120)	1	USNA unnamed hybrid
P. 'Umineko' × *P. incisa* (5124)	1	USNA unnamed hybrid
P. 'Umineko' × *P. incisa* (5126)	1	USNA unnamed hybrid
5124 × 5120 (5127)	1	USNA unnamed hybrid
5124 × 5120 (5128)	1	USNA unnamed hybrid
5124 × 5120 (5129)	1	USNA unnamed hybrid
5124 × 5120 (5131)	1	USNA unnamed hybrid
P. 'Dream Catcher' ('Okame' open-pollinated)	1	USNA Introduction (61050)
P. 'First Lady' ('Okame' × *P. campanulata*)	1	USNA Introduction (61051)
P. 'Kursar'	1	Glenn Dale, MD PI station (55631)
P. 'Okame' (*P. incisa* × *P. campanulata*)	1	Glenn Dale, MD PI station (18355)
P. 'Umineko' (*P. incisa* × *P. serrulata*)	1	Glenn Dale, MD PI station (1190)
P. 'Accolade' (*P. sargentii* × *P. subhirtella*)	1	USNA (69777)
P. 'Pandora' (*P. subhirtella* × *P. yedoensis*)	1	USNA (59239)
Test Hybrids (USNA unnamed hybrid populations)		
P. maackii × *P. maximowiczii*	3	Samples from these five crosses were used for testing marker inheritance only and were not included in phenogram (see Table 3).
'First Lady' × 5124	3	
P. maximowiczii × *P. sargentii*	3	
5124 × 5120	3	
P. cyclamina × *P. subhirtella* 'Autumnalis'	2	
P. ×*yedoensis* × *P. takesimensis*	2	Samples from these three crosses were used to test for maternal chloroplast inheritance (data not shown).
P. verecunda × *P.* ×*yedoensis*	3	
P. padus × *P. virginiana*	2	

[z]Original collection data are provided if known; otherwise, location of germplasm is listed. USDA = U.S. Department of Agriculture, PI = Plant Introduction, Pref. = Prefecture, WLPGR = Woody Landscape Plant Germplasm Repository.
[y]These cultivars could be classified in the "Sato-zakura group" according to the description by Jefferson and Wain (1984).

Materials and Methods

Plant materials and DNA extraction. A total of 119 species, cultivars, and hybrids, mostly from the flowering cherry breeding and germplasm collections at the USNA, was included in the study of genetic relationships. Parents and multiple progeny from eight controlled hybridizations were used to test the inheritance of markers (Table 1). Leaf material was collected from one plant for cultivar (clonal) accessions and from up to three trees (if available) for seed-derived accessions.

Total genomic DNA was extracted using the rapid one-step extraction (ROSE) method (Steiner et al., 1995). About 50 mg of fresh young leaf tissue was collected in 1.5-mL microfuge tubes containing 200 μL of ROSE buffer [10 mM Tris-HCl, pH 8.0, 312.5 mM EDTA, 1% sodium lauryl sarkosyl, and 1% polyvinylpolypyrrolidine (PVPP)] and a pinch of garnet matrix

(Bio101, Vista, CA). Tubes were placed in a 90 °C water bath for 20 min and were vortexed vigorously for 30 s halfway through the incubation period. The samples were placed on ice for 5 min to allow insoluble materials to settle. Aliquots of the resulting supernatant were diluted 170-fold and were used directly in PCR reactions.

SSR primers and PCR reactions. We tested over 20 SSR primer pairs isolated from different *Prunus* species (*P. cerasus*, *P. dulcis*, and *P. persica*) and chose five SSR primer pairs based on their scorability, repeatability with our samples, species origin, nuclear or organelle sources, and level of polymorphism. Five SSR primer pairs were used to amplify the following loci (Table 2): AB, CPDCT033, GA59, GA77, and pchgms3. Primers tested but not used included BPPCT001, BPPCT0019, and BPPCT0025 (Dirlewanger et al., 2002); ccmp2 and ccmp7 (Weising and Gardner, 1999); CPDCT006, CPDCT025, and CPDCT039 (Mnejja et al., 2005); PS08E08 and pchgms2 (Sosinski et al., 2000); PMS2, PMS67, PceGA25 and PceGA59 (Cantini et al., 2001); TPScp9 (Ohta et al., 2005); UDP96–001, UDP96–002, UDP97–403, and UDP98–411 (Testolin et al., 2000).

Primers were synthesized by Integrated DNA Technologies (Coralville, IA). PCR products were fluorescently labeled with 6-carboxy-fluorescein (FAM) using the FAM-labeled universal M13 primer in the single tube-nested PCR method, which labels the forward primer indirectly using just one labeled M13 (-21) primer (Schuelke, 2000).

DNA amplification was performed in 10 µL of total volume. For nuclear SSR primers, reactions contained 1.7 µL of diluted DNA, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 1.0 µM FAM-M13 and reverse primer, 0.25 µM of M13-forward primer, and 0.2 U of Taq DNA polymerase (Promega, Madison, WI). Reactions for the chloroplast SSR primer, AB, were identical except that the primer concentration and the quantity of DNA were reduced by half.

Nuclear SSR alleles were amplified with initial denaturation at 94 °C for 3 min, followed by 30 cycles of: 10 s at 94 °C, 30 s at the specified annealing temperature (Table 2), and 30 s at 72 °C. These cycles were followed by an additional 8 cycles of 10 s at 94 °C, 30 s at 53 °C, and 30 s at 72 °C with a final extension at 72 °C for 10 min. The chloroplast alleles were amplified using the same cycle parameters but with only 25 and 5 cycles of each profile, respectively.

Data collection. One microliter of the PCR reaction was mixed with 9.85 µL of deionized formamide and 0.15 µL of LIZ™ Size Standard (Applied Biosystems, Foster City, CA), denatured at 95 °C for 10 min, and then run on an ABI 3730 Genetic Analyzer. The size of the fluorescently labeled PCR product was calculated automatically by GeneScan 2.1 software (Applied Biosystems). Electropherograms were viewed and processed using GeneScan 2.1 and Genotyper 2.0 software (Applied Biosystems). All data were manually checked to ensure accuracy and consistency in calling allele sizes.

Data analysis. Hierarchical clustering analysis linking all samples was done using the R software (R Development Core Team, 2006). The presence-absence data were converted to a distance matrix with the MASS package using the distance algorithm developed for binary data. The unweighted pair group method using the arithmetic averages (UPGMA) clustering algorithm was used to generate a phenogram. The probability values for each cluster (the probability of a true cluster at each edge of the dendrogram), or *P* values, were computed with the add-on packages pvclust (Suzuki and Shimodaira, 2006) and scaleboot (Shimodaira, 2006) using multiscale bootstrapping with resampling (Shimodaira, 2004). Multiscale bootstrapping differs from ordinary bootstrapping in that resampling occurs with sample sizes different from the original sample size (both smaller and larger), and corrects biases that can occur using ordinary bootstrapping (Shimodaira, 2004). Default resampling sizes (60%–140% of the original sample size of 119) and 20,000 resamplings were used to estimate the *P* values.

Results and Discussion

Evaluation and inheritance of SSR markers in *Prunus*. The five SSR primer pairs generated a total of 140 alleles, with a range of 10 to 40 bands per locus, which revealed 115 unique genotypes among the 119 individuals tested. The chloroplast primer (AB) had the least number of alleles and the smallest size range (Table 2). The level of polymorphism, size range, and number of alleles seen in this study is high compared with studies using these primers in only one or several related *Prunus* species (Brettin et al., 2000; Cantini et al., 2001; Downey and Iezzoni, 2000; Mnejja et al., 2005; Sosinski et al., 2000). However, the level of variability is similar to that seen in other flowering cherry SSR studies (Ohta et al., 2005). This variability reflects the high level of genetic diversity in section Pseudocerasus in general and in our collection in particular.

To test the inheritance of these codominant markers in our taxa, we included parents and multiple progeny from eight controlled crosses from our breeding program (Table 1, Test hybrids). The allelic inheritance at each SSR locus was compared between hybrids and their parents, with the partial data set shown, for brevity, in Table 3. Most parental alleles were transmitted to the progeny, although some were lost due to segregation of markers from a heterozygous parent. In addition, occasional nonparental alleles appeared in the hybrids. We did not sequence these nonparental bands; however, studies on new guinea impatiens (*Impatiens hawkeri*) (Parks et al., 2006)

Table 2. Marker name, origin, DNA source, annealing temperature, and number and size of alleles and references of the five SSR primer pairs used in this study.

SSR marker name	*Prunus* species origin	DNA source	Annealing temp (°C)	Alleles observed (no.)	Product size range (bp)	Sequence reference
AB	*P. cerasus*	Chloroplast	50	10	237–265	Brettin et al., 2000
CPDCT033	*P. dulcis*	Nucleus	57	30	107–158	Mnejja et al., 2005
GA59	*P. cerasus*	Nucleus	55	39	175–263	Cantini et al., 2001
GA77	*P. cerasus*	Nucleus	55	21	166–207	Bliss et al., 2002
pchgms3	*P. persica*	Nucleus	55	40	154–223	Sosinski et al., 2000

Table 3. Inheritance of SSR markers in *Prunus* hybrids from controlled interspecific crosses showing expected Mendelian inheritance and nonparental bands (in bold) in the progeny. Only a partial data set is included for brevity. All species in this table are diploid ($2n = 2x = 16$) except *P. maackii*, which is tetraploid ($2n = 4x = 32$) based on Darlington and Wylie (1955).

Cross	Plant	CPDCT033 alleles		GA59 alleles		GA77 alleles		pchgms3 alleles[z]	
		Parental	Hybrid	Parental	Hybrid	Parental	Hybrid	Parental	Hybrid
P. maackii ×	1	141 × (127, 132)	127, 141	(237, 241) × (200, 206)	206, 237	(180, 186) × (176, 190)	186, 190	(180, 188, 198) × (177, 190)	180, 188, 190
P. maximowiczii	2		127, 141		200, 241		176, 186		177, 180, 198
	3		132		206, 241		186, 190		180, 190, 198
'First Lady' × 5124	1	143 × 138	143	(208, 249) × (202, 208)	208, 249	(180,186) × (176, 186)	180, 186	(178, 199) × (180, 186)	180, 199
	2		143		208, 249		186		178, 180
	3		143		208, 249		186		186, 199
P. maximowiczii ×	1	(127, 132) × 134	127, 134	(200, 206) × (200, 202)	202, 206	(176, 190) × (176, 180)	**186**, 190	(177, 190) × (176, 178)	177, 178
P. sargentii	2		**131**, 134		202, 206		176		190, 178
	3		127, 134		200, 206		176		190, 176
5124 × 5120	1	138 × (132, 143)	143	(202, 208) × (204, 249)	204, 208	(176, 186) × (180, 186)	ND	(180, 186) × (178, 188)	186
	2		**139**, 143		208, 249		ND		178, 186
	3		**139**, 143		208, 249		176, 180		178, 180
P. cyclamina ×	1	(136, 143) × 134	**132**, 143	200 × (204, 206)	200, 204	(182, 188) × (184, 186)	182, 184	ND	ND
P. subhirtella 'Autumnalis'	2		**121**, 134		200, 206		**178**, **180**	ND	ND

[z]ND = no data due to unclear bands (primer GA77 in hybrid 5124 × 5120) or primers not tested on that cross (primer pchgms3 in hybrid *P. cyclamina* × *P. subhirtella*).

showed that nonparental alleles ranging in size from the insertion/deletion of a single basepair to multiple copies of the single repeat unit were all related based on sequence homology. In our study, we speculate that the 1-bp difference in alleles between the hybrid and parents, as seen with SSR locus CPDCT033, could be due to insertions/deletions upstream or downstream from the repeat region. While a single base difference can also be attributed to analysis errors caused by a wide bin width, the presence of the paternal marker just 3 or 4 bp away makes this explanation unlikely with this marker. With the GA77 locus, the alleles of hybrid *P. cyclamina* × *P. subhirtella* 'Autumnalis' (bottom row) are 4 bp (twice the single repeat unit) smaller than the parental markers. Such a difference could be due to deletions as described above or may be the result of a stepwise mutation (Valdes et al., 1993). Nonparental bands such as those seen in progeny #2 of this cross could also be due to mislabeling of seedlings or pollen contamination.

Robustness of clustering. Different distance metrics and clustering algorithms can result in different clustering of accessions. Usually such differences are minor, but can cause some individuals to move across major clusters. We chose our clustering method based on the relatively high bootstrap probabilities compared with those produced using other algorithms, as well as the size and nature of the data set. The standard errors of the bootstrap *P* values were less than 0.05 for all except nine (of 117 total) clusters. Thus, we have high confidence that splits between clusters with high *P* values are real; likewise, splits between clusters with low *P* values are likely artificial and could be "moved up" to the next level. The *P* value for one node (Fig. 1, cluster K) was zero.

Genetic relationships among *Prunus* subgenera and sections. Although our primary intent was to evaluate the diversity of ornamental taxa in the subgenus *Cerasus* section *Pseudocerasus*, we also included accessions from another section in the subgenus as well as four other subgenera of *Prunus* (Table 1). The relationship of these accessions, based on UPGMA clustering, is shown in Fig. 1. As expected based on the taxonomic classifications, the ornamental cherries (sections *Pseudocerasus* and *Phyllomahaleb*) used in this study cluster together (cluster separated at node A) and were relatively removed from members of other subgenera and sections. *Prunus maackii* forms a distinct cluster (D) nearer subgenus *Cerasus* than other members of subgenus *Padus* examined in this study. Phylogenetic studies using ITS nuclear ribosomal sequences alone (Lee and Wen, 2001) or with chloroplast sequences (Bortiri et al., 2006) also placed *P. maackii* closer to members of subgenus *Cerasus*. This species, commonly known as the Manchurian or Amur cherry, is native to China, Korea, and Russia (USDA, 2009) and has leafy racemose flower clusters typical of subgenus *Padus*. Bortiri et al. (2006) hypothesized that this character state is the result of convergent evolution, which has lead to taxonomic problems in resolving relationships between subgenera *Padus* and *Laurocerasus*. *Prunus maackii* is a tetraploid ($2n = 4x = 32$), while most of the taxa in subgenus *Cerasus* are thought to be diploid (Darlington and Wylie, 1955). Despite genetic distance and ploidy differences, interspecific hybrids between *P. maackii* and members of subgenus *Cerasus* have been successfully created in our program (Table 3). These hybrids were verified as triploids in our laboratory using flow cytometry (data not shown), and are seed- and pollen-sterile, as expected.

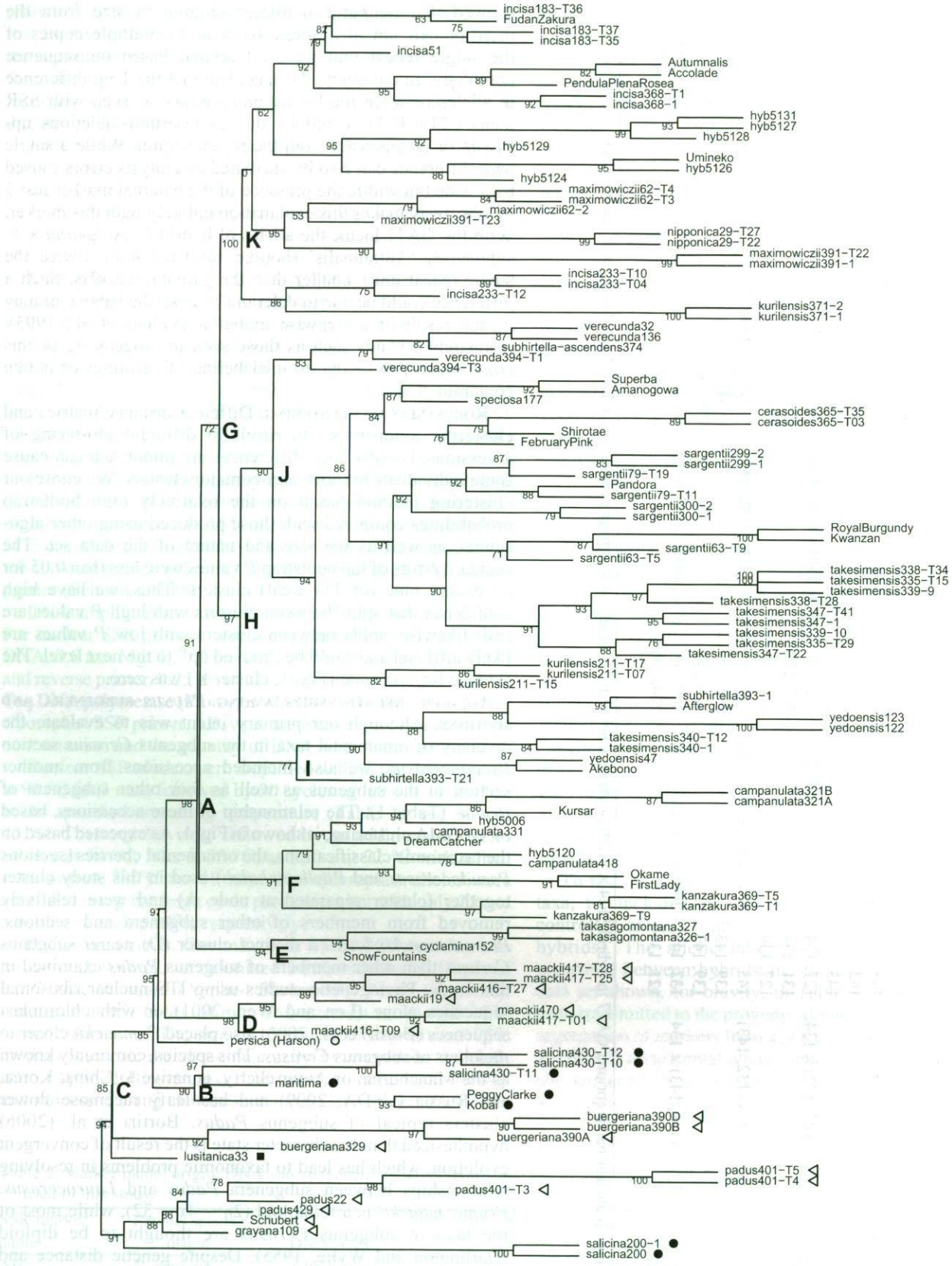

Fig. 1. Phenogram of 119 *Prunus* species, hybrids, and cultivars. Symbols designate subgenus and/or section, as defined in Table 1. Letters to the right of branch nodes are provided for reference in the text. Numbers to the left of (below) branch nodes are bootstrap *P* values for that node.

Interestingly, members of subgenus *Prunus* (*P. mume*, *P. salicina*, and *P. maritima*) clustered somewhat closer (cluster B) to flowering cherries than the remaining taxa of subgenera *Padus* and *Laurocerasus* (cluster C). This unexpected clustering within subgenera could be an artifact of the markers used, as SSR markers are most effective at differentiating individuals in the same or closely related species (Powell et al., 1996), whereas sequence analysis may be more appropriate to study relationships at higher levels. Such a conclusion is supported by higher-order phylogenetic studies of *Prunus* using ITS sequences (Bortiri et al., 2001; Lee and Wen, 2001) that seek to resolve the relationship between subgenera in *Prunus*.

The placement of one accession of *P. salicina* (200) outside the clade containing the other members of the subgenus *Prunus* was also unexpected. We suspect, however, that this accession is misidentified, as it was received as seed collected from a cultivated plant in Canada, whereas our other accession of *P. salicina* (430) was wild-collected in Shaanxi, China, and likely is a true representative of this species.

Relationships among the ornamental cherries (subgenus *Cerasus*, section *Pseudocerasus*). Using UPGMA clustering, accessions of ornamental cherries in subgenus *Cerasus* grouped into one large cluster (A) composed of several clusters that generally correspond with known taxonomic associations. These clusters are useful not only in assessing relative genetic distances among taxa, but may also be valuable in identifying mislabeled accessions or determining origins of cultivars or hybrids.

The most distant clusters in the section *Pseudocerasus* include a small cluster containing *P. takasagomontana*, *P. cyclamina*, and *P.* 'Snofozam' (Cluster E), and the larger cluster (F) containing accessions of *P. campanulata* and several of its known or proposed hybrids, including *P.* (*P. incisa* × *P. campanulata*) 'Okame' and *P.* (*P. nipponica* var. *kurilensis* × *P. campanulata*) 'Kursar' (Ingram, 1970); *P.* (*P. incisa* × *P. campanulata*) 'First Lady' and 'Dream Catcher' (Pooler, 2007); and hybrids 5006 and 5120 (Table 1). The accession *P.* ×*kanzakura*, which is a probable hybrid between *P. campanulata* and *P. serrulata* var. *lannesiana* (Flower Association of Japan, 1982) also clustered in this group. The relatively distant clustering of *P. campanulata* from other ornamental taxa supports previous studies of ornamental *Prunus* taxa by Ohta et al. (2005). In our study, this distance likely reflects different geographic origins as well because the *P. campanulata* as well as the *P. takasagomontana* in nearby cluster E were all collected from Taiwan. Although *P. campanulata* forms a group that is distinct from other taxa in this section, it crosses readily with other ornamental *Prunus* species to form fertile hybrids (Ingram, 1970; Pooler, 2007).

The remaining ornamental taxa form one large cluster G that can be further divided into smaller groups, clusters H and K. Cluster H consists of two groups, the *P.* ×*yedoensis* group (I) and a larger group containing primarily *P. takesimensis*, *P. sargentii*, and *P. verecunda* (J). The well-defined clustering (I) of *P.* ×*yedoensis* accessions in this study may reflect their common origin from Japan. Previous studies (Roh et al., 2007) indicated that *P.* ×*yedoensis* germplasm from Korea is distinct from germplasm originating in Japan.

The *P. sargentii* samples included in this study (forming a branch in cluster J) consisted of eight plants from four accessions, and were grown from seed collected from three different locations in Japan. The *P. takesimensis* were grown from seed collected from Ullung Island, Korea. It is therefore interesting to note that the *P. sargentii* accessions clustered closest to a clade containing the *P. takesimensis* accessions, despite their diverse origins. These taxa formed a larger cluster that includes 'Kwanzan' and 'Royal Burgundy' and a still larger cluster (J) that includes taxa from the Sato-zakura group (Jefferson and Wain, 1984), as well as *P. verecunda*.

We found that *P. maximowiczii* (section *Phyllomahaleb*) clustered with section *Pseudocerasus* in cluster K (primarily *P. incisa* and *P. nipponica*), which agrees with a study by Ohta et al., (2007) using chloroplast sequence data, but differs from a study using nuclear SSR markers (Ohta et al., 2005), which found *P. maximowiczii* and *P. cerasoides* accessions clustered further from the other flowering cherry taxa than other groups. These results could be due to different markers used or, more likely, different accessions used in each study. The accessions used in our study were collected as seed from Japan (maximowiczii62) and Korea (maximowiczii391). Interestingly, the Korean samples clustered closest to a *P. nipponica* accession collected as seed in Japan (see below).

Identity and clustering of specific accessions in this study. In addition to providing information on species relationships, these SSR markers were also useful in identifying germplasm in our collection that may have been misidentified or of questionable origin. For example, one accession of *P. takesimensis* (340) clustered with *P.* ×*yedoensis* in cluster I, rather than in cluster J with other *P. takesimensis* accessions. Although all accessions of this species were collected in 1986 from Ullung Island, Korea, our records indicate a questionable identity of accession 340 after seed germination. SSR genotyping confirmed a more likely identity or possible parentage of this accession.

The position of 'February Pink' in cluster J was unexpected based on this cultivar's identification as *P. incisa* (Jacobsen, 1996) or *P. nipponica* var. *kurilensis* (Kuitert, 1999) (cluster K). The accession originally came to the U.S. Plant Introduction Station, Glenn Dale, MD, from Hillier and Sons Nursery in Winchester, UK, in 1956, and cuttings were received by the USNA in 1981. It is possible that the cuttings were mislabeled during one of these transfers or that the original plant was misidentified. Alternatively, 'February Pink' is a hybrid of *P. incisa* and another species. If the first split in cluster K is ignored (*P* value of 0 at main node in cluster K) then cluster K has two subclusters encompassing most *P. nipponica*- and *P. incisa*-related taxa; 'February Pink' is clearly not included in this group.

Accessions of one other species, *P. nipponica*, also did not cluster according to accession/collection labels. Accession 371 (cluster K), collected in 1982 from cultivated material in Hokkaido, Japan, was identified as *P. nipponica* var. *kurilensis*. This accession clustered distantly from accession 211 (cluster J), which was collected in 1974 in Hokkaido and also identified as *P. nipponica* var. *kurilensis*. Accession 29 (cluster K), which was collected as seed in 1979 in Japan and identified as *P. nipponica*, clustered distantly from the other two accessions. Such results indicate possible misidentification of these accessions in our collection or, more likely, suggest that outcrossing by different species has contributed to this open-pollinated seed.

The scattered placement of six accessions or hybrids of *P. subhirtella* in the phenogram points to the nomenclatural ambiguity surrounding this taxon and its conspecific taxa. *Prunus subhirtella* is thought to be a hybrid between *P. pendula*

f. *ascendens* and *P. incisa* (Flower Association of Japan, 1982; Kuitert, 1999) or a distinct species (Ingram 1929) with *P. pendula* f. *ascendens* listed as a synonym for *P. subhirtella* var. *ascendens* (USDA, 2009). The two cultivars of *P. subhirtella* used in this study, 'Autumnalis' and 'Pendula Plena Rosea', clustered close to each other in the *P. incisa*/*P. maximowiczii* cluster (K). Another accession (393), received as seed from the Flower Association of Japan, clustered near *P.* ×*yedoensis* accessions (I). One accession of *P. subhirtella* var. *ascendens* (= *P. pendula* f. *ascendens*), which is thought to be a parent of *P.* ×*yedoensis* (Innan et al., 1995; Takenaka, 1963, 1965), clustered with *P. verecunda* in cluster J. Finally, the clustering of purported hybrids of *P. subhirtella*, 'Pandora' (cluster J) and 'Accolade' (cluster K), further point to the diversity or nomenclatural confusion of this taxon. Studies of *P. subhirtella* cultivars and germplasm using these or other molecular markers could help to elucidate the origin of this species as well as its contribution to ornamental cherry germplasm.

Inheritance of the chloroplast SSR marker AB. Previous studies have found that chloroplast-specific markers are useful in *Prunus* phylogenetic analyses (Ohta et al., 2005, 2007). Analysis of sour cherry progenies (Brettin et al., 2000) indicated that the chloroplast genome is maternally inherited in *Prunus*. We confirmed maternal inheritance of the chloroplast AB SSR marker by examining parents and progeny of three controlled crosses of ornamental flowering cherry from our breeding program (Table 1; marker data not shown). Across all *Prunus* accessions studied, this primer produced 10 alleles ranging in size from 237 to 265 bp, which gave rise to nine different *Prunus* haplotypes. This marker was particularly useful in separating taxa in section *Laurocerasus*. Unique single-allele genotypes were seen for *P. buergeriana*, where four individuals from two accessions had a single 248-bp allele. Similarly, *P. salicina* var. *mandshurica* had a single 257-bp allele. Genotypes of other species in this section were more complex. Five individuals from three accessions of *P. padus* displayed five chloroplast AB alleles, which defined three genotypes. One allele (247 bp) present in all *P. padus* accessions was also present in *P. grayana* and *P. virginiana* 'Schubert'.

While the majority of the ornamental flowering cherry taxa (subgenus *Cerasus*) contained just a single 251-bp chloroplast allele and could not be differentiated by this marker, there were several notable exceptions. The five *P.* ×*yedoensis* selections and one *P. subhirtella* var. *ascendens* used in this study displayed a 260-bp allele, which was also shared by *P. lusitanica* and an accession that was originally acquired as *P. takesimensis* (340), but, as stated previously, is likely misidentified. *Prunus* ×*yedoensis* is thought to be a hybrid between *P. pendula* f. *ascendens* (=*P. subhirtella* var. *ascendens*) and *P. lannesiana* var. *speciosa* (=*P. speciosa*) (Innan et al., 1995; Takenaka, 1963, 1965). This unique and conserved 260-bp shared marker supports the role of *P. subhirtella* var. *ascendens* as a maternal parent of *P.* ×*yedoensis* (Kaneko et al., 1986). This and other chloroplast markers (Ohta et al., 2007) may lead to new insights into the origin of *P.* ×*yedoensis*.

Placement of hybrids (controls) and cultivars within the tree. Several hybrid selections of known parentage from the USNA flowering cherry improvement program were included in the study as controls for SSR marker inheritance and to test the reliability of the clustering methodology. Generally, these hybrids clustered as expected based on known parentage. Hybrids tended to cluster closer to the maternal parent, possibly because of a bias caused by the maternally inherited chloroplast marker, or possibly, in some cases, because the pollen parent could be misidentified, while the seed parent is certain. Cluster K included two hybrids (5124 and 5126) that resulted from a controlled hybridization of *P.* 'Umineko' and *P. incisa*. Both of these hybrids clustered near *P.* 'Umineko'. Similarly, hybrids 5127, 5128, 5129, and 5131 represent a four-way cross of (*P.* 'Umineko' × *P. incisa*) (5124) × (*P. subhirtella* 'Autumnalis' × *P. campanulata*) (5120). All four of these selections clustered near each other and near the maternal parent within cluster K. Interestingly, the male parent in this cross (5120) clustered distantly in the *P. campanulata* cluster (F).

The location in the tree of several hybrid cultivars of known parentage was also well predicted. For example, 'Umineko', a hybrid between *P. incisa* and *P. speciosa* (Ingram, 1929) clustered near other *P. incisa* accessions (cluster K), but distantly from the one *P. speciosa* accession tested (cluster J). *Prunus* 'Kursar', a hybrid between *P. nipponica* var. *kurilensis* and *P. campanulata* (Ingram, 1970) clustered within the *P. campanulata* accessions (cluster F). Similarly, *P.* 'Dream Catcher', 'First Lady', and 'Okame', which contain *P. campanulata* ancestry (Ingram, 1970; Pooler, 2007), all clustered in that branch. In most cases, the individual parental trees from which these cultivars were derived are not available for testing, therefore inheritance of specific SSR alleles cannot be traced. However, the clustering of these hybrids near known parental species provides evidence that these SSR markers can be useful in determining parentage or genetic makeup of unknown flowering cherry hybrids.

Application to germplasm conservation and use. SSR markers have proven to be a powerful tool in assessing genetic diversity and accession identity in this diverse collection of flowering cherries and relatives. By testing the inheritance of these markers in controlled crosses and including known interspecific hybrids as controls in the cluster analysis, we have confidence that the results accurately portray the genetic diversity and identity of the accessions in our collection. It should be noted, however, that extrapolation of these results to flowering cherry taxonomy should be done with caution. Our collection represents only a small portion of the flowering cherry taxa, and the number of accessions used for each species or taxa was small. In addition, many of the accessions in our collection were received as open-pollinated seed collected from wild or cultivated trees primarily in Japan and Korea. While the identity of the seed parent was usually clear, the pollen parent was unknown, and in some cases could have been of another species. Long-term flowering cherry breeding work at the USNA and elsewhere has shown that interspecific hybrids among flowering cherry taxa are easy to create, thus, some of the accessions that we received as open-pollinated seed could actually be interspecific hybrids. This possibility is supported by the documentation of naturally occurring interspecific hybrid cherry taxa in Japan (Flower Association of Japan, 1982). An additional issue is the number and representativeness of markers used. Ideally, variable SSRs should be sufficiently numerous across all levels in the phenogram to construct a reliable distance matrix. However, because the accession relationships are, in theory, not well established before an analysis, one cannot a priori select the most appropriate

markers. Thus, it is important that the markers used are representative of the genome, or that they are sufficiently numerous to generate reproducible results. Because relatively few markers (compared with the size of the genome) were used, error in placement of some accessions in the phenogram, due to just one or two unusual alleles, could also be responsible for some of the discrepancies we noted when interpreting the phenogram.

The data on genetic distances provided by this study will be useful in breeding strategies aimed at developing disease- and pest-resistant hybrids, combining different ornamental traits, and increasing diversity of ornamental *Prunus*. The flowering cherry collection at the USNA represents one of the most diverse collections in the United States, including germplasm from Japan and Korea such as *P. takesimensis*, *P. takasagomontana*, and *P. cyclamina*, that have not contributed significantly to the flowering cherry gene pool. In addition to breeding with underused species, it may also be possible to increase diversity through wide hybridizations. For example, we have successfully crossed *P. maackii* with *P. campanulata*, *P. maximowiczii*, *P. subhirtella*, and *P.* 'Kursar'. However, these hybrids are seed and pollen sterile, and will therefore have to undergo chromosome doubling to be useful in further breeding. Overcoming crossing barriers using chromosome doubling (Contreras and Ranney, 2007) or embryo rescue (Liu et al., 2007) may also lead to novel species combinations, including possible hybrids from different sections.

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